US007101842B2

(12) **United States Patent**
Faulk

(10) **Patent No.: US 7,101,842 B2**
(45) **Date of Patent: Sep. 5, 2006**

(54) **TARGETED DELIVERY OF DRUGS FOR THE TREATMENT OF PARASITIC INFECTIONS**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 166 days.

(21) Appl. No.: **10/477,992**

(22) PCT Filed: **May 16, 2002**

(86) PCT No.: **PCT/US02/11893**

§ 371 (c)(1),
(2), (4) Date: **Nov. 17, 2003**

(87) PCT Pub. No.: **WO02/091992**

PCT Pub. Date: **Nov. 21, 2002**

(65) **Prior Publication Data**

US 2004/0161428 A1 Aug. 19, 2004

Related U.S. Application Data

(60) Provisional application No. 60/291,017, filed on May 16, 2001, provisional application No. 60/291,018, filed on May 16, 2001.

(51) **Int. Cl.**
A61K 38/00 (2006.01)
A61K 31/70 (2006.01)

(52) **U.S. Cl.** **514/2**; 514/8; 514/21; 514/34; 514/52; 530/300; 530/394

(58) **Field of Classification Search** 514/8, 514/21, 34, 2, 52; 530/380, 392, 394, 395, 530/406, 410, 807, 828, 300; 536/6.4
See application file for complete search history.

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(57) **ABSTRACT**

Targeting agents such as transferrin and transcobalamin can be conjugated with anti-protozoan drugs for the treatment of protozoan infections. Any suitable anti-protozoan drug can be used, preferably the drug is selected from the group consisting of apoptosis inducing compounds, cytotoxic antibiotics, alkalating agents, plant toxins, and bacterial mutant toxins. The targeting agent is preferably coupled to the antiprotozoan drug by means of glutaraldehyde.

15 Claims, No Drawings

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TARGETED DELIVERY OF DRUGS FOR THE TREATMENT OF PARASITIC INFECTIONS

CROSS REFERENCE TO RELATED APPLICATION

This application is a 35 USC § 371 National Phase Entry Application from PCT/US02/11893, filed May 16, 2002, and designating the U.S, which claims priority benefit of U.S. Provisional Application Nos. 60/291,017 filed May 16, 2001 and 60/291,018 filed May 16, 2001. The disclosure of the International Application and the two U.S. Provisional Applications are hereby incorporated by reference.

FIELD OF THE INVENTION

This invention relates generally to the field of bio-affecting materials and, more specifically to bio-affecting materials suitable for treating cells that are infected with a parasite.

BACKGROUND OF THE INVENTION

Protozoa are unicellular eukaryotic organisms that can infect and multiply in mammalian hosts. They may utilize more than one type of host, including insect hosts, during their life cycle. Parasitic protozoa account for a significant portion of all infectious diseases worldwide. Although the majority of protozoan infections occur in developing countries, these infections are seen increasingly in industrialized countries among immigrants and immunosuppressed or immunodeficient individuals. Commonly seen parasitic diseases include malaria, trypanosorriasis, and Chagas disease. The treatment of protozoan infections is problematic due to lack of effective chemotherapeutic agents which traverse the blood brain barrier, excessive toxicity of the therapeutic agents and increasingly widespread resistance to the therapeutic agents. Well known and presently used drugs for treating parasitic infections, caused by protozoa include the drugs melarsopral, eflornithine, chloroquine, quinine, mefloquine, amodiaquine, primaquine, pyrimethamine, sulfadoxine, sulfadiazine, trimethoprim, pentavalent antimony, pentamidine, amphotericin-B, rifampin, metronidazole, ketoconazole, benznidazole, nifurtimox, and halofantrinc.

Two common problems in treatments which involve drugs are drug-toxicity, which debilitates patients, and drug-resistance, which requires more drugs and thus amplifies the problem of drug-toxicity, often resulting in death. One way to solve the problem of drug-toxicity is to deliver drugs so they are targeted only to the infected cells or tissues. Many researchers are working to develop antibodies to deliver drugs, and this approach holds promise, but antibodies are not without problems. For example, they often cross-react with normal tissues, and they can damage blood vessels (e.g., vascular leak syndrome) and cause dangerous allergic reactions (e.g. anaphylaxis).

The treatment of specific cells by the delivery of drugs, including drugs that are toxic to such cells, is not new. U.S. Pat. Nos. 4,886,780; 4,895,714; 5,000,935; and 5,108,987 to Faulk and U.S. Pat. No. 4,590,001 to Stjernholm et. al., describe cytotoxic. or radioimaging materials conjugated to proteins, mainly to transferrin, as treatments for cancerous cells or for imaging cancerous cells.

It is known that stressed cells, such as, for example, human cells hosting a parasitic infection, call for an increased delivery of nutrients, such as iron, by presenting

an increased number of receptors for nutrient carriers, such as transferrin in the case of iron. The increase in receptors for nutrient carriers in stressed cells is known to be relatively constant and orders of magnitude greater in number than in unstressed cells, which are known to show receptors intermittently and in relatively smaller numbers. The publications listed above, and others, disclose taking advantage of the increased number of receptors, especially for transferrin, presented by cancer containing cells to deliver imaging materials or drugs or both to the stressed cell.

No single study has asked if all stressed cells have up regulated transferrin receptors, or if all normal cells have down regulated transferrin receptors, but data from many quarters suggest that all normal cells have down regulated transferrin receptors. For example, immature erythrocytes (i.e., normoblasts and reticulocytes) have transferrin receptors on their surfaces, but mature erythrocytes do not (Lesley J, Hyman R, Schulte R and Trotter J. Expression of transferrin receptor on murine hematopoietic progenitors. Cell Immunol 1984; 83: 14–25). Circulating monocytes also do not have up regulated transferrin receptors (Testa U, Pelosi E and Peschle C. The transferrin receptor. Crit Rev Oncogen 1993; 4: 241–276), and macrophages, including Kupffer cells, acquire most of their iron by a transferrin-independent method of erythrophagocytosis (Bothwell T A, Charlton R W, Cook J D and Finch C A. *Iron Metabolism in Man*, Blackwell Scientific, Oxford, 1979). In fact, in vivo studies indicate that virtually no iron enters the reticuloendothelial system from plasma transferrin (for review, see Ponka P and Lok C N. The transferrin receptor: role in health and disease. Int J Biochem Cell Biol 1999; 31: 1111–1137.). Macrophage transferrin receptors are down regulated by cytokines such as gamma interferon (Hamilton T A, Gray P W and Adams D O. Expression of the transferrin receptor on murine peritoneal macrophages is modulated by in vitro treatment with interferon gamma Cell Immunol 1984; 89: 478–488.), presumably as a mechanism of iron-restriction to kill intracellular parasites (Byrd T F and Horowitz M A. Interferon gamma-activated human monocytes downregulate transferrin receptors and inhibit the intracellular multiplication of *Legionella. pneumophila* by limiting the availability of iron J Clin Invest 1989; 83: 1457–1465.).

In resting lymphocytes, not only are transferrin receptors down regulated, but the gene for transferrin receptor is not measurable (Kronke M, Leonard W, Depper. J M and Greene W C. Sequential expression of genes involved in human T lymphocyte growth and differentiation. J Exp Med 1985; 161: 1593–1598). In contrast, stimulated lymphocytes up-regulate transferrin receptors in late G_1 (Galbraith R M and Galbraith G M. Expression of transferrin receptors on mitogen-stimulated human peripheral blood lymphocytes: relation to cellular activation and related metabolic events. Immunology 1983; 133: 703–710). Receptor expression occurs subsequent to expression of the c-myc proto-oncogene and following up-regulation of IL-2 receptor (Neckers L M and Cossman J. Transferrin receptor induction in mitogen-stimulated human T lymphocytes is required for DNA synthesis and cell division and is regulated by interleukin 2. Proc Nat Acad Sci USA 1983; 80: 3494–3498.), and is accompanied by a measurable increase in iron-regulatory protein binding activity (Testa U, Kuhn L, Petrini M, Quaranta M T, Pelosi E and Peschle C. Differential regulation of iron regulatory element-binding protein(s) in cell extracts of activated lymphocytes versus monocytes-macrophages. J Biol Chem 1991; 266: 3925–3930), which stabilizes transferrin receptor mRNA (Seiser C, Texieira S and Kuhn L C. Interleukin-2-dependent transcriptional and

post-transcriptional regulation of transferrin receptor-mRNA. J Biol Chem 1993; 268: 13,074–13,080.). This is true for both T and B lymphocytes (Neckers L M, Yenokida G and James S P. The role of the transferrin receptor in human B lymphocyte activation. J Immunol 1984; 133: 2437–2441), and is an IL-2-dependent response (Neckers L M and Trepel J B. Transferrin-receptor expression and the control of cell growth Cancer Invest 1986; 4: 461–470).

Malaria

Approximately 40% of the world's population are at risk for malaria. That is, in excess of 2000 million people in about 100 countries are at risk (Gilles, 1991, World Health Organization, Geneva). Particularly affected are children in developing countries (Greenwood et al., Trans Soc Trop Med Hyg 1987; 81:478). For example, a million children die of malaria every year in sub-Saharan Africa (World Health Organization, 1974, Technical Report Series No. 537). The rise of travel, trade and tourism also has extended malaria into developed countries (Greenberg & Lobel, Ann Intern Med 1990; 113:326). These social and economic problems are compounded by the complexities of vector control and the problematic development of an effective malaria vaccine (Graves & Gelband, Cochrane Database of Systematic Reviews CD000129, 2000). Thus, anti-malarial drugs remain the bulwark of defense against malaria, but this is being eroded by the spreading emergence of drug resistant strains of *Plasmodiurn falciparurn*, causing safe, widely available and inexpensive drugs like chloroquine to be increasingly less effective (Clyde, Epidemiol Rev 1987; 9:219). Taken together, these observations-indicate a pressing need for new drug strategies in the war on malaria The present invention provides a new strategy for the design of anti-malarial drugs.

The *Plasmodium falciparum* parasite reproduces rapidly within red blood cells of its host. Red cells are invaded by the merozoite stage of the parasite, which matures into the trophozoite stage and sufficiently replicates its DNA to produce **32** daughter cells within 48 hours. Like all developing cells (Richardson & Ponka, Biochim Biophys Acta 1997; 1331:1), developing plasmodia require iron to promote the function of key enzymes, such as ribonucleotide reductase for DNA synthesis (Chitambar et al., Biochem J 2000; 345:681), and iron-dependent enzymes for pyrimidine synthesis, CO_2 fixation and mitochondrial electron transport (Mabeza et al., Acta Haematol 1996; 95:78). The importance of iron in plasmodial development has been demonstrated in both in vitro Cabantchik et al., Acta Haematol 1996; 95:70) and in vivo (Pollack et al., Proc Soc Exp Biol Med 1987; 184:162) models in which growth of parasites is inhibited by iron chelation. The most widely studied iron chelator is deferoximine, which is a siderophore or chelator that tightly (i.e., affinity of 10^{31}/M) binds iron (Peto & Thompson, Br J Haematol 1986; 63:273). Clinical studies of Zambian children with advanced cerebral malaria (e.g., comatose) have revealed that patients treated with a standard program of anti-malarial therapy plus deferoxamine (100 mg/kg/day) recovered more rapidly than patients who received the same program of anti-malarial therapy without deferoxarine (Gordeuk et al., N Engl J Med 1992; 327:1473).

In light of the key role played by iron in the growth and development of plasmodia, much research has focused on how plasmodia obtain iron, and whether the parasites can be killed by drugs that interfere with the metabolic pathways that are used to acquire iron. Conceptually, plasmodia can obtain iron either from within the red blood cells in which they reside, or from the patient's transferrin, which is the normal protein in blood that carries iron (Ponka & Lok, Int J Biochem Cell Biol 1999; 31:1111). There is little doubt that plasmodia are capable of obtaining iron from red blood cells (Hershko & Peto, J Exp Med 1988; 168:375). In order to obtain iron from the patient's transferrin, there must be transferrin receptors on red blood cells, but normal adult red blood cells do not manifest transferrin receptors (Richardson & Ponka, Biochim Biophys Acta 1997; 1331:1). However, malaria infected red blood cells bind transferrin (Pollack & Fleming, Br J Haematol 1984; 58:289), and data have been produced that have identified 102 kD (Haldar et al., Proc Natl Acad Sci USA 1986; 83:8565) and 93 kD (Rodriguez & Jungery, Nature 1986; 324:388) transferrin receptors in the plasma membranes of red blood cells infected with *Plasmodium falciparum*. Although these observations have been challenged (Pollack & Schnelle, Br J Haematol 1988; 68: 125), subsequent experiments have shown that the receptors are functional, inasmuch as they have been used to deliver an anti-plasmodial toxin to infected red cells, and such delivery was inhibited by antibody to transferrin (Surolia & Misquith, FEBS Letters 1996; 396:57).

Trypanosomiasis

Trypanosomiasis is a parasitic infection caused by trypanosomes, which are protozoans that are passed to human beings by the bite of an infected tsetse fly (Smith et al., Brit Med Bull 1998; 54:341). When introduced into patients, trypanosomes proliferate in blood and lymphatics, which is the first stage of disease; the second stage of disease develops when parasites traverse the blood-brain-barrier and cause neurological damage and lethargy, commonly known as sleeping sickness (Beutivoglio et al., Trends Neurosci 1994; 17:325). If untreated, trypanosomiasis in both humans and animals is a fatal disease (New York Times, May 21, 2000).

There are two clinical forms of infection that are caused by different trypanosome subspecies. First, *Trypanosoma brucei* gambiense causes a chronic disease that takes several years to reach advanced stage; second, *Trypanosoma brucei* rhodesiense causes an acute disease that is fatal within weeks; Both diseases are endemic in Africa, and infections with *Trypanosoma brucei* gambiense currently are epidemic, placing at risk 60 million people inhabiting 36 sub-Saharan countries (Barrett, Lancet 1999; 353:1113). In addition, trypanosomiasis is limited neither to Africa (Dissanaike, Celyon Med J 2000; 45:40) nor to humans (Karnau et al., Prevent Vet Med 2000; 44:231), and the economic impact of these diseases profoundly impact national economies (Bauer et al., Trop. Animal Hlth & Prod 1999; 31:89).

Diagnostic approaches to trypanosomiasis have been designed to identify the stage of disease in patients, for early infections limited to blood and lymphatics can be treated with less toxic drugs than later infections involving the central nervous system (Dumas & Buiteille, Med Trop 1997; 57:65). There are currently two drugs for treatment of central nervous system infections (i.e., sleeping sickness). The least expensive, most available and most toxic is melarsopral, which is an arsenical drug that induces a fatal encepholopathy in 5–7% of recipients (Harrison et al., Am J Trop Med Hyg 1997; 56:632). These problems are compounded by drug resistance, low response rates and relapse rates as high as 10% (Pepin & Milard, Adv Parasitol 1994; 33:1). A less toxic, more expensive and difficult to acquire alternative to melarsopral is eflornithine, which is an ornithine decarboxylase inhibitor that impedes polyamine syn-

thesis (Sjoerdsma & Schechter, Lancet 1999; 354:254), but this molecule presently is being marketed as an expensive anti-cancer drug.

There also currently are two drugs available for treatment of early stage infections. One of these, pentamidine, was developed in 1941, and the other, suramin, was developed in 1920. Pentamidine also is effective in *Pneumocystis carinii* infections common in AIDS patients, and it is about 4-fold more expensive than suramin, which for the moment is used only in trypanosomiasis. There are other compounds with trypanocidal activity (Enanga et al., Trop Med Int Health 1998; 3:736), but most of these do not cross the blood-brain-barrier and thus are of limited usefulness in infections of the central nervous system.

The targeted delivery of drugs has the advantage of increasing efficacy while using less drug, thereby decreasing toxicity and causing less damage to normal cells, all of which effectively decrease costs and increase the quality of patient care. Targeted delivery also avoids drug-resistance, which is activated by the non-specific entrance of drugs into cells (Marbeuf-Gueye C, Ettori D, Priebe W, Kozlowski H and Gamier-Suillerot A. Correlation between the kinetics of anthracycline uptake and the resistance factor,in cancer cells expressing the multidrug resistance protein or the P-glycoprotein. Biochem Biophy Acta 1999; 1450: 374–384). Because transferrin-drug conjugates enter cells specifically by employing a receptor-specific pathway (Klausner R D, vanReuswoude J, Ashwell G, Kempf C, Schechter A N, Dean A and Bridges K. Receptor-mediated endocytosis of transferrin in K562 cells. J Biol Chem, 1983; 258: 4715–4724.; Berczi A,.Ruthner M, Szuts V, Fritzer M, Schweinzer E and Goldenberg H. Influence of conjugation of doxorubicin to transferrin on the iron uptake by K562 cells via receptor-mediated endocytosis. Euro J Biochem 1993; 213: 427–436.), they are trafficked around drug-resistance mechanisms, such as efflux pumps in resistant cells.

There exists an unfulfilled need for an inexpensive and effective agent for selectively targeting and eliminating cells diseased by protozoan parasitic invasion

SUMMARY OF THE INVENTION

The present invention provides a material for treating parasitic protozoa infections such as malaria, trypanosomiasis, and Chagas disease (which can be caused by *Trypanosomo cruzi*). The material is a conjugate comprising a targeting agent such as transferrin or transcobalamin and an anti-protozoan drug. Suitable drugs include but are not limited to doxorubicin, deferoxamine, melarsopral, eflornithine, pentamidine, quinine, mefloquine, amodiaquine, primaquine, pyrimethalrnme, sulfadoxine, sulfadiazine, trimethoprim, pentavalent antimony, amphotericin B, rifamnpin, metronidazole, ketoconazole, benznidazole and nifurtimox, and suramin. The present invention also provides a method for treating patients infected with a protozoa and a composition containing the conjugate.

DETAILED DESCRIPTION OF THE INVENTION

The above discussed needs are filled by a conjugate for treating infected cells, especially cells stressed by a protozoan infection, that, in one embodiment includes a targeting agent that is attracted to a receptor that is expressed in higher numbers or more frequently by cells infected by a protozoa than by normal uninfected cells, and an anti-protozoan drug.

The targeting agent can be any material that is attracted to receptors on cells that present in higher numbers Or more frequently when a cell is stressed from a protozoan infection. Preferably, the targeting agent is transferrin.

Attachment of the drug to the targeting agent may be by any mechanism that prevents their separation, at least until after the targeting agent has been positioned in the corresponding receptor. Presently, the best known mechanism for attachment for a transferrin-doxorubicin conjugate is a gluteraldehyde linker, but the linker can be any material useful for the targeting agent/drug combination in question.

Technical details of the conjugation procedure can vary, but the requirement of any procedure is to prepare defined conjugates that are (a) active in binding and killing experiments with protozoan infected cells, and that (b) do not bind or kill significant numbers of normal cells. In light of these requirements, when transferrin is used as the targeting agent and doxorubicin is used as the anti-protozoan drug, the preferred method for preparing the conjugates according to the present invention is the following process.

The synthesis of large amounts of homogeneous transferrin-doxorubicin conjugates with predetermined molecular ratios was done stoichiometrically by employing the only amino group of doxorubicin (DOX), which is at the 3' amino position, to react with one of the two reactive groups on glutaraldehyde (GLU). Thus, the first step was drop-wise addition of a saline solution of DOX into a saline solution of GLU containing a solvent such as DMSO or another suitable cryopreservative, to a final concentration of a 1:1 molar ratio of DOX-to-GLU. The resulting solution of DOX-GLU was stirred three hours at room temperature in the dark.

The molarities of DOX and GLU were the same in the above reaction in order to produce a final solution of DOX-GLU that contains neither free DOX nor free GLU. However, there is the possibility of free GLU in solution if one GLU reacts with two DOX to produce DOX-GLU-DOX, but this possibility is minimized by the mass action kinetics generated by drop-wise addition of monovalent DOX into the solution of bivalent GLU. The volumes of these reactants are not restricted, so large amounts of homogeneous DOX-GLU can be prepared.

The second step in the conjugation reaction was drop-wise addition of DOX-GLU into a saline solution of transferrin (TRF). The TRF can be either iron-free (apo-transferrin) or iron-saturated (holo-transferrin). The desired molar ratio of DOX to TRF was obtained by appropriately adjusting the volume of TRF. The resulting solution of TRF-GLU-DOX was stirred for 20 hours at room temperature in the dark. Unlike the reaction of DOX with GLU, the reaction of DOX-GLU with TRF is not restricted to one binding site, for the GLU component of DOX-GLU can react with any one of several epsilon-amino lysine groups in the TRF molecule.

The number of DOX molecules bound to TRF was determined in the second step. For example, if the starting ratio of DOX-GLU to TRF was 7.2:1.0, the final solution of TRF-GLU-DOX would have contained 2.5 molecules of DOX per molecule of TRF. However, if the starting ratio of DOX-GLU to TRF was 4.0:1.0, the final solution of TRF-GLU-DOX would have contained 1.4 molecules of DOX per molecule of TRF. Similarly, if the starting ratio of DOX-GLU to TRF was 2.5:1.0, the final solution of TRF-GLU-DOX would have contained 0.9 molecules of DOX per molecule of TRF. In this way, large amounts of TRF-GLU-DOX with predetermined ratios of DOX-to-TRF can be provided according to the need.

One skilled in the art will appreciate that there may be unreacted linker and a small amount of unintended constructions, such as DOX-GLU-DOX in the reaction, product and that it will be desirable to optimize the reaction product by removing them. Ethanolamine or another substance suitable for scavenging any excess linker may be added to the reaction product, followed by centrifugation and dialysis, may be used to remove excess GLU and such unintended constructions. Although reactions with DOX and TRF theoretically consume all of the GLU, ethanolamine was added to the final reaction mixture to bind any available GLU. This reaction was allowed to continue for 30 minutes in the dark. The final solution was centrifuged at 2000 rpm for 10 minutes, dialyzed twice for 6 hours in a 100-fold excess of saline and three times in the same excess of Hepes buffered saline, and the resulting TRF-GLU-DOX conjugates were ready for use.

Biochemical Characterization of the Conjugates:

By using HPLC and polyacrylamide gel electrophoresis, the homogeneity of TRF-GLU-DOX conjugates can be determined. Also, by using spectrophotometry, the molecular ratio of DOX-to-TRF can be determined. These techniques repeatedly have revealed a consistent homogeneity of the TRF-GLU-DOX conjugates. In addition, chromatography is not required in the preparation of these conjugates, because there are no aggregates or fragments. This allows for the preparation of large volumes of homogeneous transferrin-drug conjugates, which increases yields and decreases costs.

The expenses caused by losses of TRF and DOX in other types of transferrin-drug conjugates have been an impediment to their use. For example, yields of DOX and TRF are decreased by using procedures such as thiolation that alter the drug and/or protein. Yields also-are decreased by using solvent systems and by chromatography used to prepare acid-stable and acid-labile linkages. The GLU bond between DOX and TRF is acid-stable, and yields of useful conjugates prepared according to this invention are high Indeed, compared to other procedures, the yield for useful conjugate is increased 5-fold.

None of the previously known approaches to the preparation of transferrin-doxorubicin conjugates are capable of producing large amounts of homogeneous conjugates with predetermined ratios of the number of drug molecules per molecule of transferrin. In addition, the known approaches employ chromatography to eliminate aggregates and to harvest fractions that are enriched in homogeneous conjugates. These procedures decrease yields, increase costs, and lack the ability to predetermine molecular ratios.

After the conjugates are isolated, they can optionally be characterized by polyacrylamide gel electrophoresis to determine their molecular weight, and the number of drug molecules per protein molecule can be determined. Experience with drug-protein conjugates in other systems has shown that a functional drug:protein ratio is 0.1–4.0 molecules of drug per molecule of protein (Berczi et al., Arch Biochem Biophy 1993; 300:356), recent unpublished data-suggest that lower conjugation numbers are still significantly cytotoxic, while higher conjugation numbers (e.g., >4.0) tend to be associated with unstable conjugates. Other steps in the characterization of the conjugates are to (a) determine if the conjugates bind to transferrin receptors on the surface of infected cells and not uninfected cells, and (b) determine if the conjugates kill protozoan infected cells and not uninfected cells. The binding studies can be done by using flow cytometry, and the killing studies can be done by using microculture techniques to determine the concentration of free drug required to kill 50% of a culture of infected cells compared to the concentration of drug in the drug-protein conjugate required to kill the same number of infected cells. Experience with drug-protein conjugates in other systems indicates that approximately 10-fold more free drug compared to the drug in drug-protein conjugates should be required to kill the same number of infected cells. For example, the dosage of a conjugate of transferrin-doxorubicin is expected to be between 0.5–50 mg per 28 day period for a 150 pound (68 kg) person The dosage can be administered as smaller doses at varying intervals during the 28 day period. For a conjugate to be efficacious, preferably it should kill none or only a minimum of uninfected cells.

Treatment of Malaria

Since drug resistance (World Health Org., Technical Report Series No 692, 2000) and drug toxicity (Winstanley, J Roy Col Phy London 1998; 32:203) are major problems in the treatment of malaria, the aim of the present invention is to provide a ligand-receptor method for the targeted delivery of anti-malarial drugs designed to utilize the pathways employed by plasmodia to acquire iron In one embodiment, the ligand is human transferrin, the receptor is plasmodial transferrin receptor, and the drug is either the cytotoxic drug doxorubicin which also is an iron chelator (Myers, Seminars Oncol 1998; 25:10); or the iron-chelating siderophore deferoxamine (also known as desferrioxamine or Desferal). Deferoxarnine is a hydroxamate-based hydrophilic chelator of iron (Tsafack et al., J Lab Clin Med 1996; 127:574). The molecule has a terminal NH_2 that has been derivatized with molecules such as nitrobezyl-diazole and N-methylanthranile without reducing its property of iron chelation (Loyevsky et al., J Clin Invest 1993; 91:218).

Treatment of Trypanosomiasis

A carrier is needed that could transport trypanocidal compounds across the blood-brain-barrier. The normal plasma protein transferrin has been shown to accomplish this task by means of interacting with transferrin receptors on endothelial cells that compose the microcirculation of the blood-brain-barrier (Broadwell et al., Exp Neurol 1996; 142:47). For example, a conjugate of transferrin with nerve growth factor has been shown to be transported from blood into the brain (Li et al., J Natural Tox 2000; 9:73), and the object of the present invention is to provide conjugates of trypanocidal drugs with transferrin that can be transported from blood across the blood-brain-barrier into the central nervous system, thereby providing effective therapy for both early and late stages of trypanosomiasis.

In addition to being an effective transporter of trypanocidal drugs across the blood-brain-barrier, transferrin can be targeted to transferrin receptors present on trypanosomal plasma membranes (Borst et al., Science 1994; 264:1872). Like human transferrin receptors, trypanosomal receptors are regulated post-transcriptionally by iron (Fast et al., Biochem J 1999; 342:691). Each trypanosome contains about 3000 receptors, which are heterodimers linked by a glycosylphosphatidylinositol anchor to the plasma membrane where they concentrate in flagellar pockets, among a sea of variant surface glycoprotein (Borst & Fairlamb, Ann Rev Microbiol 1998; 52:745). Trypanosomes require iron, which they obtain from the transferrin of their host (Schell et al., EMBO J 1991; 10:1061). Since they can thrive in many different mammalian hosts, and since transferrins differ in different mammals, trypanosomes have about 20 gene copies of transferrin receptors, which allows them to produce a high-affinity receptor to bind and internalize host

transferrins, whether they be in animals or human patients (Bitter et al., Nature 1998; 391:499). Thus, intravenously administered trypanocidal drug conjugates of transferrin circulate throughout the body, including the central nervous system, where they are bound by trypanosomal transferrin receptors and exercise their trypanocidal properties.

The present invention is a drug-protein conjugate which can be used for the targeted delivery of a cytotoxic drug to trypanosomes in infected patients, whether they are in early or late stages of disease, and regardless of which Trypanosoma species with which they are infected. Targeted delivery of drugs is possible in this invention because the preferred protein in the drug-protein conjugate is transferrin, which is relevant because trypanosomes have transferrin receptors on their surfaces (Bitter et al., Nature 1998; 391:499). In addition, the drug in the drug-transferrin corrugate can be a known trypanocidal agent, or cytotoxic drug such as doxorubicin. While being present on the surfaces of cancer cells (Yeh et al., Vox Sang 1984; 46:217), transferrin receptors usually are not present on the surface of normal, adult, resting cells (Berczi et al., Arch Biochem Biophy 1993; 300:356). Thus, most normal cells in trypanosomiasis patients will not be affected, and the only cells to be eliminated by cytotoxic transferrin conjugates will be the trypanosomes, whether they are in blood, lymph or the central nervous system.

One way to illustrate targeted drug delivery to trypanosomes in patients is to focus on the use of transferrin, which carries iron in the blood. Transferrin can be obtained by isolation from blood plasma, from commercial suppliers, or from recombinant technology (Ali et al., J Biol Chem 1999; 274;2406 6). To form the drug-protein conjugate, transferrin molecules must be modified in such a way as to prepare them to be coupled with a trypanocidal or cytostatic drug. The drug can be an arsenical such as melarsopral, a cytotoxic antibiotic such as doxorubicin or an inhibitor of polyarnine synthesis such as eflomithine, but any compound can be used, including plant toxins such as ricin, and bacterial mutant toxins such as modified diphtheriatoxin (Laske et al., Nature Med 1997; 41:1039).

Several coupling processes such as glutaraldehyde coupling (Yeh & Faulk, Clin Immunol Immunopathol 1984; 32:1), disulfide coupling (Sasaki et al., Jap J Can Res 1993; 84: 191) or benzyl hydrazine coupling (Kratz et al., J Pharm Sci 1998; 87: 338) have been used to couple transferrin with other molecules. The wide variety of coupling procedures allows the conjugation of a broad range of drugs to transferrin, resulting in either permanent or dissociable bonding of the drugs with the transferrin molecule (Barabas et al., J Biol Chem 1992; 267:9437). Following the coupling reaction, drug-protein conjugates can be separated from uncoupled drug and free protein, if necessary by using chromatographic procedures or selective dialysis.

While the present invention has been described in relation to transferrin being the delivery protein, it is known that other proteins exist in the body which are capable of binding to receptor sites on infected cells. If the receptor site is activated in infected cells, and is inactive in uninfected cells, then any protein or other compound which binds to such a receptor site can be used to deliver the drugs used in the present invention. One example of such a binding protein is transcobalamin, which delivers vitamins, especially vitamin B12, to transcobalamin receptors on cells in the human body (Seetheram, Ann Rev Nutr 1999; 19:173). Other examples include but are not limited to ceruloplasmin, vitamin binding proteins, hormones, cytokines, low density lipoproteins, and growth factors.

The conjugates according to the present invention are administered to an animal in an effective amount In treating protozoan infections, an effective amount includes an amount effective to reduce the amount of protozoa The dosage for the conjugates can be determined taking into account the age, weight and condition of the patient and the pharmacokinetics of the anti-protozoan agent. The amount of the conjugate required for effective treatment will be less than the amount required using the anti-protozoan agent alone and depends upon the anti-protozoan agent used. For example, the dosage of a conjugate of transferrin-doxorubicin is expected to be between 0.5–50 mg for a 150 pound (68 kg) person. The dosage can be administered as smaller doses at varying intervals and repeated if necessary.

The pharmaceutical compositions of the invention can be administered by a number of routes, including but not limited to orally, topically, rectally, ocularly, vaginally, by pulmonary route, for instance, by use of an aerosol, or parenterally, including but not limited to intramuscularly, subcutaneously, intraperitoneally, intra-arterially or intravenously. The compositions can be administered alone, or can be combined with a pharmaceutically-acceptable carrier or excipient according to standard pharmaceutical practice. For the oral mode of administration, the compositions can be used in the form of tablets, capsules, lozenges, troches, powders, syrups, elixirs, aqueous solutions and suspensions, and the like. For parenteral administration, sterile solutions of the conjugate are usually prepared, and the pHs of the solutions are suitably adjusted and buffered. For intravenous use, the total concentration of solutes should be controlled to render the preparation isotonic. For ocular administration, ointments or droppable liquids may be delivered by ocular delivery systems known to the art such as applicators or eye droppers. For pulmonary administration, diluents and/or carriers will be selected to be appropriate to allow the formation of an aerosol. It is preferred that the conjugate of the present invention be administered parenterally, i.e. intravenously or intraperitoneally, by infusion or injection.

As used in the present document, the term "substantially homogeneous conjugates" means that the conjugates can be used without further purification to remove protein dimers, polymers or aggregates. In other words, little or no protein dimers, polymers or aggregates are present.

Preferred embodiments of the present invention are described below. It will be apparent to those of ordinary skill in the art after reading the following description that modifications and variations are possible, all of which are intended to fall within the scope of the claims.

EXAMPLE 1

Preparation of Conjugates

The synthesis of large amounts of homogeneous transferrin-doxorubicin conjugates with predetermined molecular ratios was done stoichiometrically by employing the only amino group of doxorubicin (DOX), which is at the 3' amino position, to react with one of the two reactive groups on glutaraldehyde (GLU). The first step was to add GLU drop-wise to DMSO in an ice cold water bath. Next was the drop-wise addition of a saline solution of DOX into a saline solution of GLU+DMSO to a final concentration of a 1:1 molar ratio of DOX-to-GLU. The resulting solution of DOX-GLU was stirred three hours at room temperature in the dark.

The molarities of DOX and GLU were the same in the above reaction in order to produce a final solution of

DOX-GLU that contains neither free DOX nor free GLU. However, there is the possibility of free GLU in solution if one GLU reacts with two DOX to produce DOX-GLU-DOX, but this possibility is minimized by the mass action kinetics generated by drop-wise addition of monovalent DOX into the solution of bivalent GLU. The volumes of these reactants are not restricted, so large amounts of homogeneous DOX-GLU can be prepared.

The second step in the conjugation reaction was drop-wise addition of DOX-GLU into a saline solution of transferrin (TRF). The TRF can be either iron-free (apo-transferrin) or iron-saturated (holo-transferrin). The desired molar ratio of DOX to TRF was obtained by appropriately adjusting the volume of TRF. The resulting solution of TRF-GLU-DOX was stirred for 20 hours at room temperature in the dark. Unlike the reaction of DOX with GLU, the reaction of DOX-GLU with TRF is not restricted to one binding site, for the GLU component of DOX-GLU can react with any one of several epsilon-amino lysine groups in the TRF molecule.

The number of DOX molecules bound to TRF was determined in the second step. For example, if the starting ratio of DOX-GLU to TRF was 7.2:1.0, the final solution of TRF-GLU-DOX would have contained 2.5 molecules of DOX per molecule of TRF. However, if the starting ratio of DOX-GLU to TRF was 4.0:1.0, the final solution of TRF-GLU-DOX would have contained 1.4 molecules of DOX per molecule of TRF. Similarly, if the starting ratio of DOX-GLU to TRF was 2.5:1.0, the final solution of TRF-GLU-DOX would have contained 0.9 molecules of DOX per molecule of TRF. In this way, large amounts of TRF-GLU-DOX with predetermined ratios of DOX-to-TRF can be provided according to the need.

In an optimization of the production of the conjugate, ethanolamnine is added, followed by centrifugation and dialysis. Although reactions with DOX and TRF theoretically consume all of the GLU, ethanolamine was added to the final reaction mixture to bind any available GLU. This reaction was allowed to continue for 30 minutes in the dark. The final solution was centrifuged at 2000 rpm for 10 minutes, dialyzed twice for 6 hours in a 100-fold excess of saline and three times in the same excess of Hepes buffered saline, and the resulting TRF-GLU-DOX conjugates were ready for use.

The invention claimed is:

1. A method for selectively treating a cell infected with a protozoa, comprising

administering to said cell an anti-protozoan effective amount of a conjugate containing a protozoan infected cell targeting agent and an anti-protozoan drug, wherein said anti-protozoan drug is selected from the group consisting of an apoptosis inducing compound, a cytotoxic antibiotic, an alkylating agent, a plant toxin, and a bacterial mutant toxin, and wherein said protozoan infected cell targeting agent is selected from the group consisting of transferrin and transcobalamin.

2. The method according to claim **1**, wherein said anti-protozoan drug is selected from the group consisting of doxorubicin, deferoxamine, melarsopral, eflornithine, pentamidine, quinine, mefloquine, amodiaquine, primaquine, pyrimethamine, sulfadoxine, sulfadiazine, trimethop rim, pentavalent antimony, amphotericin B, rifampin, metronidazole, ketoconazole, benznidazole, nifurtimox, suramin, ricin, and choloroquine.

3. The method according to claim **1**, wherein said protozoan infected cell targeting agent is transferrin.

4. The method according to claim **2**, wherein said protozoa is selected from the group consisting of Plasmodia species and trypanosomes.

5. A method for treating a patient infected with a protozoa, comprising administering to said patient an effective amount of a conjugate containing a protozoan infected cell targeting agent and an anti-protozoan drug, wherein said anti-protozoan drug is selected from the group consisting of an apotosis inducing compound, a cytotoxic antibiotic, an alkyating agent, a plant toxin, and a bacterial mutant toxin, and wherein said protozoan infected cell targeting agent is selected from the group consisting of transferrin and transcobalamin.

6. The method according to claim **5**, wherein said protozoa is selected from the group consisting of Plasmodia species and trypanosomes.

7. A pharmaceutical composition suitable for treating protozoan infections comprising a conjugate and a carrier, wherein said conjugate comprises a protozoan infected cell targeting agent and an anti-protozoan drug selected from the group consisting of melarsopral, eflornithine, pentamidine, quinine, mefloquine, amodiaquine, primaquine, pyrimethamine sulfadoxine, sulfadiazine, trimethoprim, pentavalent antimony, metronidazole, ketoconazole, benznidazole, nifurtimox, suramin, ricin, and choloroquine, and wherein said protozoan infected cell targeting agent is selected from the group consisting of transferrin and transcobalamin.

8. The composition according to claim **7**, further comprising an unconjugated anti-protozoan drug.

9. The composition according to claim **7**, wherein said targeting agent is transferrin.

10. A substantially homogeneous conjugate comprising a targeting agent and an anti-protozoan drug wherein said anti-protozoan drug is selected from the group consisting of melarsopral, eflornithine, pentamidine, quinine, mefloquine, amodiaquine, primaquine, pyrimethamine, sulfadoxine, sulfadiazine, trimethoprim, pentavalent antimony, metronidazole, ketoconazole, benznidazole, nifurtimox, suramin, ricin and choloroquine, and wherein said targeting agent is selected from the group consisting of transferrin and transcobalamin.

11. The conjugate according to claim **10**, wherein said targeting agent is transferrin.

12. A reagent kit for determining the susceptibility of protozoan infected cells to anti-protozoan drugs, comprising two or more conjugates each containing a protein targeting agent and an anti-protozoan drug, wherein said conjugates have different anti-protozoan drugs, and wherein at least one anti-protozoan drug is selected from the group consisting of melarsopral, eflornithine, pentamidine, quinine, mefloquine, amodiaquine, primaquine, pyrimethamine, sulfadoxine, sulfadiazine, trimethoprim, pentavalent antimony, amphotericin B, rifampin, metronidazole, ketoconazole, benznidazole, nifurtimox, suramin, ricin, and choloroquine, and wherein said protein targeting agent is selected from the group consisting of transferrin and transcobalamin.

13. A method for making a conjugate having a predetermined anti-protozoan drug: protein ratio, comprising

a) adding a solution of an anti-protozoan drug dropwise to a linker molecule solution to link each anti-protozoan drug molecule to one linker molecule in a drug/linker combination; and

b) reacting the drug/linker combination with a protein to produce a conjugate having a predetermined anti-protozoan drug: protein ratio, wherein said protein is selected from the group consisting of transferrin and

transcobalamin and wherein said anti-protozoan drug is selected from the group consisting of melarsopral, eflornithine, pentamidine, quinine, mefloquine, amodiaquine, primaquine, pyrimethamine, sulfadoxine, sulfadiazine, trimethoprim, pentavalent antimony, metronidazole, ketoconazole, benznidazole, nifurtimox, suramin, ricin, choloroquine, and deferoxamine.

14. The method according to claim **13**, further comprising scavenging any excess linker.

15. The method according to claim **13**, wherein said linker is selected from the group consisting of glutaraldehyde, benzoyl hydrazone, maleinimide and N-hydroxysuccinimide.

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